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Methods for dissecting dry insects and insects preserved in fixative solutions or by refrigeration

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The methods described in this paper for the dissection of dry and preserved insects have been used for several years on various species, mainly mosquitos. In the past, dry or partially dry mosquitos found in traps or in the laboratory had to be discarded. By softening these insects in a detergent solution, however, it is possible to make most observations in the same way as on fresh material. The preservation of insects in the dry state, in a fixative, or in the refrigerator after collection enables much larger samples to be studied; the whole of the material can be examined and the work can be done when time permits. In addition, material can be sent to central laboratories far from the place of collection, and infected insects can be kept in stock for teaching purposes.

This paper is in three sections: the first describes methods of dissection of dry insects and preservation of mosquitos in dry condition; the second deals with preservation in a fixative solution and subsequent dissection; and the third discusses methods of preserving insects by refrigeration for dissection and examination of internal organs. These methods offer new possibilities for the more complete processing of samples of vector populations collected for different studies.

THE DISSECTION OF DRY INSECTS

During field and laboratory studies a number of insects become too dry to be dissected for an examination of their internal organs. In many instances some of the insects collected in traps or experimental huts, mainly the unfed specimens or those intoxicated by an insecticide, are found dead and partially dry in the morning. A complete processing of the catch, which is particularly important for small samples, is thus impossible. In order to overcome this difficulty, a simple method for examining and dissecting dry insects was developed. The method, which has been used for 3 years in dissecting dry *Anopheles*, *Culex*, *Musca*, *Stomoxys*, *Tabanus*, *Glossina*, *Erystalis*, and *Simulium*, makes it possible not only to study the abdominal appearance of insects but also to examine the internal organs and parts of the body and to look for external and internal parasites. Repeated observations were carried out on different dried wild and laboratory mosquitos.

Rehydration

The principle of the method is to rehydrate the dry insects. The materials needed are: (1) liquid detergent, without enzymes; (2) physiological saline for invertebrates (0.65%); (3) tap water.

In the absence of a convenient detergent any type of emollient solution can be used: 1% soap solution, 0.5% sodium benzoate, a weak solution (0.5%) of potassium or sodium hydroxide, or even physiological saline alone. When physiological saline is used alone the softening time is much longer; the insects should be kept in a refrigerator at about 4°C for 12–24 hours according to their size and the degree of dryness.

Diluted solutions of almost all chemicals with detergent or emollient properties can be employed, the time needed for rehydration being established by preliminary trials for each species of insect and softening solution. Household liquid detergents may also be used, the only condition being that they should not contain enzymes. The concentration of the detergent may vary from 0.5% to 20% according to the type used and the speed of softening required.

Softening procedure

The softening process consists of two steps:

(1) The insects are immersed in a diluted detergent, one part liquid detergent to 4–9 parts water; they should be kept in this solution for 10–60 min-

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utes, according to their dryness and size and the type of solution.

(2) The insects are transferred to 0.65% physiological saline or tap water for a period varying from 20 minutes to several hours according to their size and state of dryness. When only the abdominal appearance of a mosquito is to be studied or only partially dry insects are being dissected, the insects can be prepared for examination in no more than 20 minutes by using a 20% solution of a liquid detergent and allowing 10 minutes in the detergent and 10 minutes in water.

Dissection methods

In insects that have recently been partially dried, the internal organs can be extracted after softening by using methods similar to those for fresh insects. When dealing with insects that are very dry or slightly decomposed before rehydration, the organs must be manipulated with much more care (especially when dealing with small insects) because of the considerable change in their elasticity. Methods of extracting different organs of mosquitos in such situations are illustrated in Fig. 1–3.

Extraction of salivary glands (see Fig. 1)

(1) Cut off the head and the anterior legs (A and B).

(2) Detach the area of the prothoracic segment, to which the salivary glands remain attached (C and D).

(3) Detach the salivary glands with a fine dissecting needle (E).

(4) Add a drop of 0.65% physiological saline containing a little gentian violet, methylene blue, or Giemsa stain in order to colour the tissues slightly, and examine under a cover slip at the appropriate magnification.

In some cases extraction of the salivary glands may prove very difficult if not impossible.

Extraction of the abdominal organs (see Fig. 2)

(1) Separate the abdomen from the thorax (A).

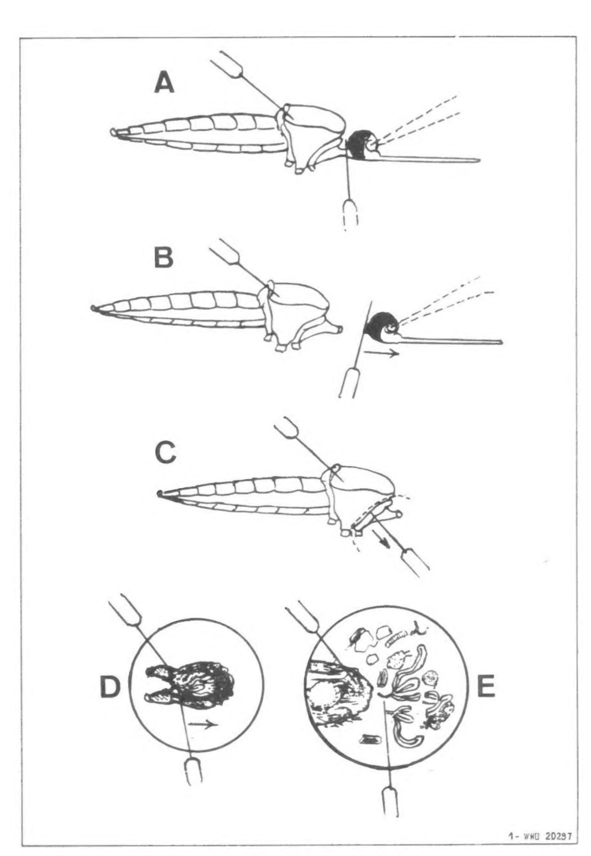


Fig. 1. Extraction of the salivary glands.

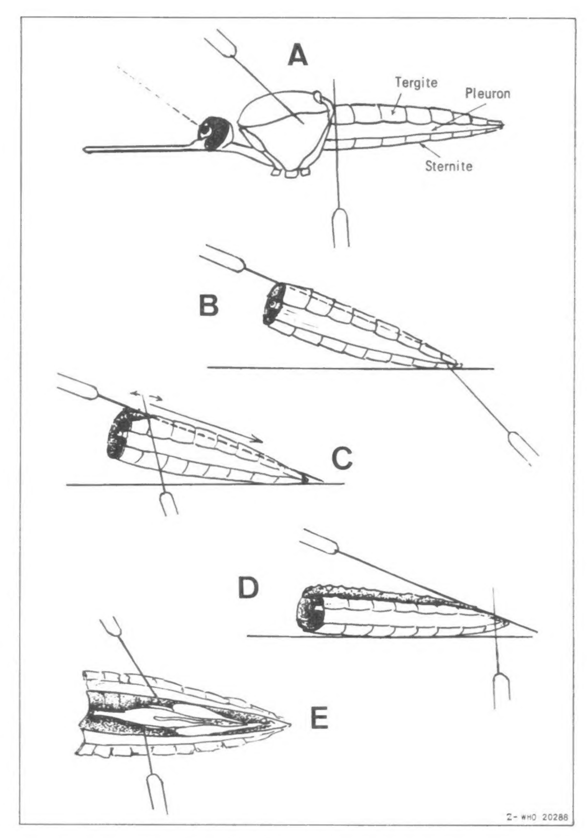


Fig. 2. Extraction of the abdominal organs.

(2) Fix the extremity of the abdomen with the right-hand needle. Insert the left-hand needle inside the abdomen along the tergal plates, advancing it slowly until it reaches the last segment and touches the slide with its tip (B).

(3) Press the right-hand needle against the left one, making a slight movement in order to cut the tergum along the whole length from segments 1 to 8 (C and D).

(4) Open the abdomen with the tips of the dissecting needles; insert one needle under the abdominal organs and remove them from the abdomen (E). If the malpighian tubes or parts of the fat body cells are covering the midgut and cannot be removed with the needle, use a very fine paint brush. Separate the ovaries and, if necessary, dissect them to examine dilatations.

(5) Add a drop of physiological saline lightly stained with methylene blue (1 : 10 000), gentian violet (1 : 10 000), or Giemsa stain (one drop to 4 ml of physiological saline) and examine on a covered slide.

Another method of opening the abdomen is illustrated in Fig. 3. The abdominal pleuron is detached with the tip of the dissecting needle (A and B) and the tergum removed (C). The organs are then separated from the rest of the abdomen (D).

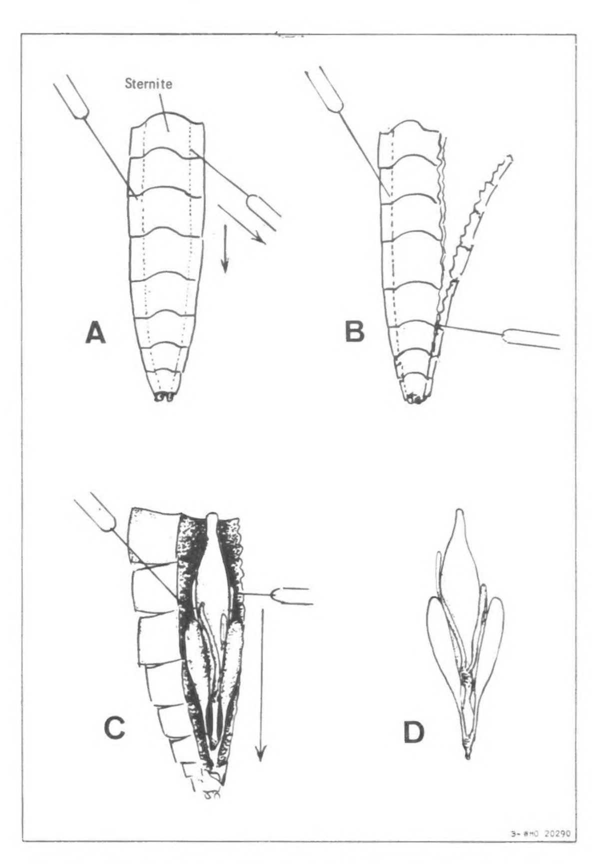


Fig. 3. Alternative method of dissecting the abdomen.

Material used and results

The rehydration method was first used under field conditions in 1968. Since then large numbers of dry mosquitos from different sources have been dissected, including the following species collected in the field: *Aedes (O.) communis* and *Ae. (O.) rusticus* (Switzerland and France), *Anopheles albimanus* (Haiti), *An. annularis* and *An. balabacensis* (Thailand), *An. funestus* and *An. gambiae s.l.* (northern Nigeria, Senegal, and Togo); *An. littoralis* (Philippines), *An. messae* (Romania and Switzerland), *An. minimus minimus* (Thailand), *An. nili* and *An. pharoensis* (Togo), *An. philippinensis* (Thailand), and *Culex pipiens* (Switzerland, France, and Romania).

Dry laboratory-bred specimens of the following anopheline species were dissected in Dr G. Davidson's laboratory at the Ross Institute of Tropical Hygiene, London School of Hygiene and Tropical Medicine, England: *Anopheles albimanus*, *An. farauti*, *An. funestus*, *An. gambiae A*, *B*, *C*, *An. labranchiae atroparvus*, *An. melas*, *An. merus*, *An. pharoensis*, and *An. stephensi*.

Dry specimens of *An. stephensi* infected with *Plasmodium berghei* were kindly provided by the Ross Institute, the Malaria Reference Centre, Horton Hospital, Epsom, England, and the Department of Parasitology, Liverpool School of Tropical Medicine, England. *Aedes albopictus* infected with *P. gallinaceum* oocysts in all stages and sporozoites was provided by the WHO International Malaria Eradication Training Centre, Manila, Philippines.

Two types of material have been dissected. The first type consisted of insects found dead and partially dried in window and light traps or in the laboratory. Secondly, insects were killed, left to dry, and then dissected after intervals of 1, 2, 3, 7, 14, 21, 30, 45, 60, 75, 180, and 300 days. After being killed the insects were transferred to small wide-mouthed bottles or to large glass or plastic tubes containing a layer of silica gel or calcium chloride

covered with a layer of cotton wool (Fig. 4). The containers were then carefully closed and sealed with solid paraffin. In a warm and dry climate the insects dry in a very short time, but to preserve them in good condition they should be placed in tubes containing silica gel or calcium chloride or in a desiccator. In a hot and humid climate, their internal organs may be rapidly disintegrated by bacteria, though the ovaries are more resistant than the digestive tract.

At the Ross Institute in April 1970, mosquitos dried in paper cups near an electric heater for 1–2 hours after killing were successfully dissected and examined for Sella's and Christophers' stages and for parity and nulliparity up to 180 days. *An. funestus*, *An. gambiae A*, and *An. melas* from Togo were kept for 45 days in containers with silica gel and then examined for abdominal appearance (Sella's stages), parity rates, stomach infections (in empty ones), condition of salivary glands, Christophers' stages, retained eggs, and refeeding by gravid females. *Agamodistomum* sp. was found in *An. funestus* from Togo and *An. m. minimus* from Thailand.

After a batch of dried *An. funestus* and *An. gambiae* from Kano, Nigeria, had been kept at room temperature in Geneva for 2 years, it proved possible to examine the insects for abdominal appearance, Christophers' stages, refeeding by gravid females, retained eggs, and *Coelomomyces*. The reactions produced by aquatic mites and by black spores were also clearly identifiable. The stomachs of unfed mosquitos can be satisfactorily examined for the presence of oocysts in all stages if well preserved in the dry state. When *An. stephensi* infected with *P. berghei* sporoblasts and sporocysts was rehydrated and dissected after being kept dry for 14 days, the fine internal structure of unstained sporoblasts could be seen and the sporozoites were visible.

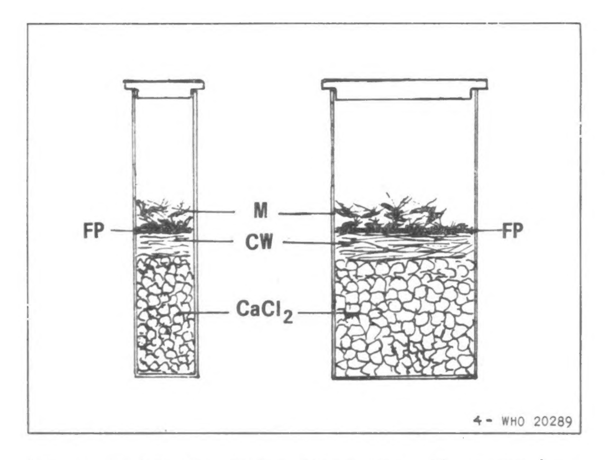


Fig. 4. Tubes for drying mosquitos. M: mosquitos; CW: cotton wool; FP: filter paper.

Apart from being dried in tubes containing silica gel or calcium chloride, mosquitos may be lyophylized under vacuum in a test-tube which is then sealed by heat, or they can be dried in a refrigerator at about 0°C. Dry mosquitos kept in the deep-freeze compartment of a refrigerator can be held for months before rehydration for dissection.

When rehydrated mosquitos cannot be dissected the same day, they can be kept in a refrigerator at a temperature ranging from −4°C to +4°C; a disinfectant (0.5% sodium benzoate) may be added to the physiological saline if a refrigerator is not available.

This method enabled dry mosquitos to be examined for the following: abdominal appearance (Sella's stages); Christophers' stages; parous and nulliparous females; sacs and dilatations in the ovarioles (mainly in recently dried specimens); refeeding of gravid females; number of mature eggs per batch; retained eggs; state of spermatheca; presence of *Coelomomyces* spores in the ovaries; mermithid larvae; filaria larvae (*Dirofilaria, Microfilaria*); encysted forms of trematode larvae; *Crithidia*; young and old *Plasmodium* oocysts in unfed mosquitos and, on a few occasions, sporozoites in the glands; and aquatic mite larvae and the reactions produced by them and by black spores.

EXAMINATION AND DISSECTION OF MOSQUITOS PRESERVED IN DIFFERENT FIXATIVE SOLUTIONS

The use of formalin or ethanol for preserving specimens of insects for identification is widespread. However, the method has not so far been employed to preserve adult mosquitos for subsequent examination of abdominal appearance and physiological stages or for the study of internal organs for various purposes (mainly in unfed mosquitos).

Method of fixation

Recently killed or anaesthetized mosquitos are dipped in one of the following solutions.

formula (a)

formalin (neutralized)	10 ml
glycerol	4 ml

acetic acid, glacial	1 ml
physiological saline 0.65 % (or distilled water)	100 ml
sodium tetraborate (10 H_2O)	0.5 g

formula (b)

ethanol, 75 %	95 ml
glycerol	5 ml

formula (c) (Bouin's solution)

picric acid, saturated aqueous solution	30 ml
formalin	10 ml
acetic acid, glacial	2 ml

Bouin's solution is particularly useful for histological studies as the cell nuclei are well fixed and their structure appears clearly.

formula (d) (Carnoy's solution)

chloroform	30 ml
ethanol, absolute	60 ml
acetic acid, glacial	10 ml

Carnoy's solution is particularly useful for fixing the chromosomes of larval salivary glands, the ovaries or malpighian tubes of adults, or malaria parasites, since it fixes the nucleus and its constituents. When fixing larval glands or adult abdominal organs, the thorax of larvae or the abdomen of adults should be separated from the rest of the body to permit rapid penetration of the fixative; alternatively, the organs should be dissected before fixing. For longer preservation, the material should be placed in solution (e) after fixation for 3–4 hours in Carnoy's solution.

formula (e)

formalin (neutralized)	6 m
physiological saline (0.65 %)	94 m

formula (f)

formalin	10 ml
water	140 ml
sodium tetraborate (10 H_2O)	0.5 g

Before dipping the mosquitos in solutions (a), (c), (e), or (f), they should first be dipped for 1–2 seconds in 90% ethanol in order to reduce the surface tension of the cuticula and help the fixative to penetrate. Many other fixative solutions may be used if desired.

Method of dissection

Before mosquitos preserved in formalin or ethanol are dissected, they should be washed for a few minutes in tap water. The internal organs of preserved mosquitos cannot be extracted by the methods used to dissect fresh mosquitos; a procedure similar to those illustrated in Fig. 1 and 2 for dissecting dried insects after rehydration should be used. Internal organs can be stained with haemalum or haematoxylin and mounted as a permanent preparation in Canada balsam or euparol. Stomachs infected with *Plasmodium* oocysts can be prepared in paraffin for microscopical section.

Mosquitos may be kept for long periods in these solutions, and can be examined even after several years for the characteristics and infections already mentioned. *Plasmodium* sporozoites can be detected in the salivary glands of these insects when stained; however, they are not as clear as in fresh specimens because the sporozoites are already fixed inside the cells of the glands.

DISSECTION OF FROZEN MOSQUITOS

Methods of preservation and dissection

Mosquitos recently collected by pyrethrum spray and live mosquitos anaesthetized by ether may be immersed in a mixture of water and glycerol in the proportion of 8 : 3 by volume in small glass or plastic bottles or tubes. This mixture has a low freezing point and mosquitos can be frozen at between −15° and −18° C while the mixture remains liquid. They should be immersed only after the mixture has reached these temperatures.

The mosquitos can be extracted one by one from the mixture while they are still frozen without defreezing the whole batch of mosquitos. After some days, however, the organs may be hardened by the glycerol.

A preferable method is to dip recently killed mosquitos for 1 second in 80% ethanol, immerse them in water in small plastic tubes, and freeze them at −18° to −25°C. The frozen mosquitos can be dissected in the same way as fresh ones, but their organs are less elastic than when fresh. Before dissection the mosquitos must be dipped in 0.65% saline.

All internal organs have been extracted in good condition after freezing for 1 month and oocysts and sporozoites can be easily detected. After 90 days, ovaries of *Aedes rusticus* were easily dissected for dilatations of the ovarioles. Observations on the practical uses of this method are continuing.

CONCLUSION

The methods described above are not intended to replace those now in use for the processing of fresh material. However, they offer the possibility in special situations of processing samples more completely. Dry insects that were previously discarded as useless, with the result that information was lost, can be dissected and examined. Mosquitos can also be preserved in a dry state, in fixative solution, or by deep freezing for later examination when lack of time and staff prevents complete processing of very large samples when fresh.

The methods permit preserved material to be sent to different laboratories for demonstration purposes and can assist in controlling the quality of work carried out by field teams. Indeed, certain aspects of the work may be undertaken at leisure, at a convenient time under proper laboratory conditions.

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RÉSUMÉ

MÉTHODES DE DISSECTION D'INSECTES DESSÉCHÉS ET D'INSECTES CONSERVÉS DANS DES SOLUTIONS FIXATIVES OU PAR RÉFRIGÉRATION

L'auteur décrit des méthodes de dissection d'insectes partiellement ou entièrement desséchés ou conservés dans différentes solutions fixatives. Des insectes congelés à −18°C ont également été disséqués soit pour étudier les organes internes soit pour constater une infection par un *Plasmodium* ou par d'autres endoparasites.

La dissection des insectes desséchés est rendue aisée après réhydratation. Des détergents liquides courants ont été utilisés, mais les détergents renfermant des enzymes ne devraient pas être employés. Le procédé de réhydratation comporte deux étapes: *a*) immersion des insectes dans une solution de détergent (1 partie pour 4–9 parties d'eau) où ils sont gardés pendant 10 à 60 min. La durée d'immersion dépend de l'état de dessèchement et de la taille des insectes, ainsi que du type de détergent; *b*) on place ensuite les insectes dans l'eau (ordinaire) et, après un lavage de 20 à 30 min, on les met dans une solution physiologique à 0,65%. (Le transfert des insectes dans des solutions physiologiques n'est pas nécessaire s'ils ne sont pas gardés plus d'une demi-heure à une heure dans l'eau). Le temps requis pour une réhydratation satisfaisante est facilement déterminé par chaque chercheur.

Cette méthode de réhydratation peut être appliquée soit aux insectes partiellement ou entièrement desséchés, collectés lors de différentes observations, soit aux insectes collectés vivants et intentionnellement desséchés pour être disséqués plus tard. A cette fin, il est conseillé de placer les insectes dans des tubes en verre ou en plastique, remplis au tiers de Silicagel ou de chlorure de calcium, et soigneusement refermés après l'opération. Il faut se souvenir que, dans un climat chaud et humide, les organes internes des insectes se décomposent assez rapidement. Au laboratoire, les insectes peuvent être conservés dans un dessiccateur. En utilisant la méthode de réhydratation, il est possible d'effectuer pratiquement tous les examens réalisés sur les insectes frais. L'examen des glandes salivaires des moustiques pour la recherche des sporozoïtes est un peu plus difficile à réaliser sur des insectes desséchés que sur des insectes à l'état frais. Des dissections de moustiques conservés dans la formaline ou dans l'alcool ont été effectuées en utilisant les méthodes illustrées par les figures 1, 2 et 3. Presque tous les examens effectués sur des moustiques à l'état frais ont pu l'être également sur des moustiques conservés dans une solution fixative, exception faite de l'examen du nombre des dilatations des ovarioles; l'identification des sporozoïtes dans les glandes salivaires est moins facile à effectuer sur des moustiques fixés que sur des préparations fraîches. Les même dissections et les mêmes examens sont possibles sur des moustiques congelés à −18°C et sur des moustiques frais.

Les méthodes décrites offrent de larges possibilités pour l'utilisation des échantillons d'insectes, les insectes partiellement ou totalement desséchés pouvant être utilisés pour différents examens. La conservation des insectes à l'état sec ou dans la formaline ou dans l'alcool, etc., offre la possibilité d'examiner plus tard des spécimens qui ne peuvent pas être examinés sur place, en raison du manque de temps ou d'un échantillonnage extrêmement abondant. Ces méthodes offrent aussi la possibilité d'exercer un meilleur contrôle de la qualité du travail des équipes isolées qui travaillent sans surveillance effective, ou d'examiner le matériel à une date plus convenable, ou dans un laboratoire très éloigné du lieu de collecte des spécimens.